Attorney Docket No. 058315/0117

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CYTOPLASMIC EXPRESSION OF ANTIBODIES, ANTIBODY FRAGMENTS AND ANTIBODY FRAGMENT FUSION PROTEINS IN *E. coli*

BACKGROUND OF THE INVENTION

The expression of functional antibodies and antibody fragments in *E. coli* is known in the prior art, but these methods require the use of signal sequences which direct polypeptide transport into the periplasm. When expression takes place in the *E. coli* periplasm, the expression yields are in the range of a few μg per liter of culture medium (Ayala et al., Bio Techniques 13, pp. 790- 799, 1992). In addition, refolding experiments are often required in order to obtain functionally active antibody fragments (such as Fab) or antigen-binding regions (such as a single chain Fv(sFv)). There is a need therefore, to develop improved methods for expressing functionally active antibodies and antibody fragments. The prior art does not teach recombinant production of antibodies or antibody fragments which can be isolated from the cytoplasm in functional form. Such molecules would be useful in the production of therapeutic agents.

Bagshawe describes a method for generating cytotoxic agents that are directed towards cancer sites, termed Antibody Directed Enzyme Prodrug Therapy (ADEPT). Bagshawe, Br. J. Cancer, vol. 60, pp. 275- 281, 1989. Using ADEPT, an antibody or antibody fragment that specifically binds to a cancer cell is fused to an enzyme that is capable of converting a non-toxic drug into a toxic drug. Only those cells to which the fusion protein is bound will be killed upon administration of the precursor of the toxic drug.

The β-glucuronidase of Escherichia coli has been well characterized biochemically and genetically. The gene (uid A) has been cloned by Jefferson et al. (PNAS vol. 83, pp. 8447 - 8451, 1986) and employed as a reporter gene for heterologous control regions.

β-Glucuronidase (β-D-glucuronoside glucuronosohydrolase, E.C. 3.2.1.31) is an acid hydrolase which catalyzes the cleavage of β-glucuronides. As a result of the mammalian glucuronidases having been intensively investigated, a variety of substances are available for histological, spectrophotometric and fluorometric analyses. This enzyme has gained new, additional importance in its use for fusion proteins for targeted tumor therapy. In this connection, human glucuronidase is used in the form of a fusion protein which contains antibodies/antibody fragments or antigen-binding regions (Bosslet et al., Br. J, Cancer, 65, 234 - 238, 1992). As an alternative to the human enzyme, it is also possible to use the homologous *E. coli* β-glucuronidase. One of the advantages of the *E. coli* β-glucuronidase is that its catalytic activity at physiological pH is significantly higher than that of the human β-glucuronidase.

In the past, it has only been possible to express antibody fragment-enzyme fusion molecules periplasmically in *E. coli*. The enzyme moiety which is used in this context is therefore always composed of periplasmic *E. coli* enzymes such as β-lactamase (Goshorn et al., Canc. Res. 53, 2123 - 2117, 1993).

An *E. coli* strain which is deficient in thioredoxin reductase (TRR), for example the strain AD 494, is capable of forming disulfide bridges in the cytoplasm and thus enzymes which are naturally secretory, for example alkaline phosphatase, can be expressed intracellularly See Derman et al., *Science*, 262:1744-1747, 1993. Derman describes the selection and isolation of TRR-deficient *E. coli* mutants.

The prior art does not teach expression of an antibody fragment-enzyme fusion molecule using a

cytoplasmic *E. coli* enzyme, such as β-glucuronidase, which is functionally active -- *i.e.*, which retains both enzymatic activity and antigen-binding ability of the antibody moiety. As a rule, functionally active expression of most antibodies or antibody fragment molecules requires defined signal sequences for exporting the expressed molecules via the endoplasmic reticulum into the culture medium (animal cells and yeast) or into the periplasm (*E. coli*). It is only in the endoplasmic reticulum or in the periplasm that the necessary oxidative conditions pertain for forming the disulfide bridges which are important for functional activity. In addition, the secretory synthesis route is often crucial for the correct three-dimensional folding of the expressed protein.

SUMMARY OF THE INVENTION

Thus, it is an object of the invention to provide a method for the production of functional antibodies and antibody fragments in *E. coli* in which the antigen-binding polypeptides can be isolated from the cytoplasm without the need for further processing such as protein folding and disulfide bond formation.

It is also an object of the invention to provide a method for the production of fusion polypeptides in *E. coli* comprising antibody or antibody fragment and an enzyme, in which the antibody or antibody fragment and the enzyme retain functionality and in which the functional fusion polypeptide can be isolated from the cytoplasm without the need for further processing such as protein folding and disulfide bond formation.

The invention relates, therefore, to processes for the recombinant expression of antibodies, antibody fragments or antibody fragment fusion molecules containing cytoplasmic mammalian or *E. coli* enzymes as fusion partners using thioredoxin reductase-deficient *E. coli* strains and subsequent isolation of the expression products from the cytoplasm.

The invention relates to the cytoplasmic expression of antibodies, antibody fragments and antibody fragment fusion molecules in *E. coli*. In particular, antibody fragment fusion molecules having an antibody moiety which is directed against tumors and an enzyme moiety which cleaves a nontoxic prodrug to give the toxic drug can be advantageously prepared in this way while retaining their respective functional properties.

Accordingly in one embodiment, the invention provides a method for producing an antibody or antibody fragment comprising:

a) transforming a thioredoxin reductase-deficient *E. coli* strain with a nucleotide molecule encoding said antibody or antibody fragment;

b) culturing said transformed *E.coli* strain to allow for expression of said antibody or antibody fragment; and

c) isolating said antibody or antibody fragment from the cytoplasm of said transformed *E.coli*.

In a further embodiment, the invention also provides a method for producing a fusion protein comprising an antibody or antibody fragment and an enzyme, said method comprising:

a) transforming a thioredoxin reductase-deficient *E. coli* strain with a nucleotide molecule encoding said fusion protein;

b) culturing said transformed *E.coli* strain to allow for expression of said fusion polypeptide; and

c) isolating said fusion polypeptide from the cytoplasm of said transformed *E.coli*.

In another embodiment, the antibody fragment used in the methods of the invention is selected from the group consisting of an Fab fragment, an Fv fragment, an sFv fragment and an $F(ab')_2$ fragment. The antibody used in the methods of the invention can be a humanized antibody. The invention further provides an embodiment wherein the antibodies used in the method of the invention can be antibodies or antibody fragment binds specifically to tumor cells.

In another embodiment, an enzyme used in the method for making a fusion protein is capable of cleaving a nontoxic prodrug to produce a toxic drug and may be a human cytoplasmic enzyme.

In a further embodiment, the fusion protein produced according to the invention comprises an antibody or antibody fragments that is capable of specifically binding to tumor cells and an enzyme capable of cleaving a nontoxic prodrug to produce a toxic drug.

In a further embodiment, the fusion protein produced according to the invention comprises a humanized antibody and a human cytoplasmic enzyme. In another embodiment, the invention comprises a fusion protein comprising *E. coli* ß-glucuronidase.

In another embodiment, the invention provides fusion proteins comprising an antibody or antibody fragment and an enzyme. The invention further provides fusion proteins produced according to the methods of the invention. In a further embodiment, a fusion polypeptide comprising *E. coli* β-glucuronidase and an antibody or antibody fragment is provided.

In a further embodiment, a nucleotide sequence encoding a fusion protein comprising an antibody or antibody fragment and an enzyme is provided. In still a further embodiment, the invention provides a nucleotide sequence encoding a fusion polypeptide comprising *E. coli* β-glucuronidase and an antibody or antibody fragment. In yet another embodiment, a nucleotide sequence encoding the amino acid sequence in Figure 5 which begins at nucleotide number 666 and ends at nucleotide number 3162 is provided. In a final embodiment, a nucleotide sequence is provided wherein said sequence is the nucleotide sequence in Figure 5 which beings at nucleotide number 666 and ends at nucleotide number 3165.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the insertion of the DNA sequence for *E. coli* ß-glucuronidase into the vector p

bluescript II KS (KS) to produce vector KS/A.c.-ß-Gluc. The sequences of the primers used to amplify the ß-glucuronidase gene are also shown.

Figure 2 shows the sequences of the primers used to amplify the antibody variable domain VH and the CH1 constant domain from the cDNA construct HC-hum-ß-glucuronidase. This Figure also shows the cloning of the VH/CH1 region into vector KS by cleavage of the HC-hum-ß-glucuronidase using XbaI and EcoRI restriction sites to produce vector KS/Fab HC.

Figure 3 shows the ligation of the XbaI/NcoI fragment from vector KS/Fab HC into vector KS/A.c.-ß-Gluc to produce vector KS/Fab HC/E.C. ß-Gluc.

Figure 4 shows the ligation of the XbaI/HindIII fragment of vector KS/Fab HC/E.C. ß-Gluc into vector pTrc 99/FabLC to produce vector pTrc99/dicistr. Fab/E.c.ß-Gluc.

Figure 5a shows the organization of vector pTrc99/dicistr. Fab/E.c.ß-Gluc.

Figure 5a shows the nucleotide and corresponding amino acid sequences for the VK and CK domain coding sequences inserted into the vector pTrc99/dicistr. Fab/E.c.ß-Gluc.

Figure 5a-5c shows the nucleotide and corresponding amino acid sequences for the VH, CH1 and *E. coli* ß-glucuronidase coding sequences inserted into the vector pTrc99/dicistr. Fab/E.c.ß-Gluc.

Figure 6 shows TSK-3000 gel chromatography of a purified fusion protein to determine the molecular weight of the fusion protein. The fusion protein eluted at 13.4 minutes, as evidenced in the peak absorbance at 280 nm. The positions of other molecular weight markers are shown.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has now been found that a antibody fragment, such as Mab BW 431/26 (an Fab molecule), Güssow & Seemann, *Methods in Enzymology*, 203:99-121 (1991), or a complete

antibody molecule, can be expressed cytoplasmically in a functionally active state, *i.e.*, while retaining its antigen-binding properties in an *E. coli* strain which is deficient in thioredoxin reductase.

Surprisingly, it has furthermore been possible to express an antibody fragment-enzyme fusion molecule composed of, for example, the cytoplasmic, non-disulfide-bridged *E. coli* enzyme β-glucuronidase and, for example, the Fab BW 431/26 which requires intramolecular cystine bridges for correct folding (Fab BW 431/26-*E. coli* β-glucuronidase) in the cytoplasm and to isolate it therefrom in functional form. At the same time, the expressed molecule was soluble and no refolding was necessary to produce a functionally active polypeptide. This opens up novel opportunities for economically producing antibodies, antibody fragments and antibody fragment-enzyme fusion molecule for therapeutic and diagnostic use.

Antibodies and Antibody Fragments

Examples of antibody fragments of the invention include (A) a "half antibody" molecule, *i.e.*, a single heavy:light chain pair, and (B) an antibody fragment, such as the univalent fragments Fab and Fab', the divalent fragment $F(ab')_2$, and a single or double chain Fv fragment. Antibody fragments according to the invention are preferably Fab fragments or antigen-binding regions such as sFv. See Plückthun and Skerra, Meth. Enzymol. 178, pp. 497 - 515, 1991. Many antibodes are known in the art. Antibodies according to the invention include human antibodies, humanized antibodies, and other antibodies known in the art.

Humanized antibodies are chimeric antibodies comprising non-human and human regions, and have reduced immunoreactivity when used therapeutically in humans. Typically, the variable domains or are of non-human origin and the constant domains are of human origin. Humanized antibodies can also be produced by inserting non-human complimentarity-determining-regions (CDRs) into

the framework of a human antibody. An antigen binding site in an antibody is made up of CDRs in the light chain and CDRs in the heavy chain. Humanized antibodies can be produced using recombinant DNA technology well-known in the art. Briefly, oligonucleotides encoding CDRs with desired antigen-recognition properties are used to replace the CDR regions in a human antibody gene. In certain instances, a mouse monoclonal antibody will have the desired antigen-recognition characteristics. These CDR-encoding regions are sequenced and oligonucleotides encoding these regions are inserted into the human antibody gene. See Güssow, *Methods in Enzymology* 203:99-121 (1991), which describes techniques well known in the art for humanization of antibodies and cloning antibody (immunoglobulin) genes.

The antibodies and antibody fragments according to the invention preferably bind specifically to malignant, cancerous, or tumorigenic cells. It is well known in the art that cancer cells often express specific antigens on their surface, and it is to these antigens that the antibodies and antibody fragments according to the invention specifically bind.

Also illustrative of an antibody fragment within the present invention is a non-peptide "mimetic," *i.e.*, a compound that mimics an epitope binding site of an antibody but that is water-soluble, resistant to proteolysis, and non-immunogenic. Conformationally restricted, cyclic organic peptides which mimic any of these antibodies can be produced in accordance with known methods described, for example, by Saragovi, *et al.*, *Science* 253: 792 (1991).

In accordance with the present invention, antibody fragments within the invention can be obtained from a antibody by methods that include digestion with enzymes such as pepsin or papain and/or cleavage of disulfide bonds by chemical reduction. Alternatively, antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer such

as those supplied commercially by Applied Biosystems, Multiple Peptide Systems and others, or they may be produced manually, using techniques well known in the art. See Geysen *et al.*, *J. Immunol. Methods* 102: 259 (1978). Direct determination of the amino acid sequences of the variable regions of the heavy and light chains of the antibodies according to the invention can be carried out using conventional techniques.

As noted, a fragment according to the present invention can be an Fv fragment. An Fv fragment of an antibody is made up of the variable region of the heavy chain (Vh) of an antibody and the variable region of the light chain of an antibody (Vl). Proteolytic cleavage of an antibody can produce double chain Fv fragments in which the Vh and Vl regions remain non-covalently associated and retain antigen binding capacity.

Double chain Fv fragments also can be produced by recombinant expression methods well known in the art. See Plückthun and Skerra, Meth. Enzymol. 178, pp. 497 - - 515 (1991), Skerra *et al.*, *Science* 240: 1038 (1988), and King *et al.*, *Biochemical J.* 290: 723 (1991). Briefly, the amino acid sequence of the variable regions of the heavy and light chains of antibodies known in the art can be obtained by direct amino acid sequencing using methods well known to those in the art. From this amino acid sequence, synthetic genes can be designed which code for these variable regions and they can both be inserted into an expression vector. Alternatively, nucleotide sequences known in the art which encode antibodies can be employed. Two polypeptides can be expressed simultaneously from a mammalian or bacterial host, resulting in formation of an active Fv fragment.

An antibody fragment of the present invention also can be a single-chain molecule or so-called "single chain antigen binding polypeptide," a phrase used in this description to denote a linear polypeptide that binds antigen with specificity and that comprises variable or hypervariable regions from the heavy and light chain

chains of an antibody. Single chain antigen binding polypeptides that retain an antigen-binding capacity that is characteristic of the present invention can be produced by conventional methodology. The Vh and Vl regions of the Fv fragment can be covalently joined and stabilized by the insertion of a disulfide bond. See Glockshuber, *et al.*, *Biochemistry* 1362 (1990). Alternatively, the Vh and Vl regions can be joined by the insertion of a peptide linker. A gene encoding the Vh, Vl and peptide linker sequences can be constructed and expressed using a recombinant expression vector. See Colcher, *et al.*, *J. Nat'l Cancer Inst.* 82: 1191 (1990). Amino acid sequences comprising hypervariable regions from the Vh and Vl antibody chains can also be constructed using disulfide bonds or peptide linkers, as described herein.

Fusion Proteins

Fusion proteins can be made in *E. coli* using recombinant DNA techniques that are well-known in the art. See, *e.g.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY pp. 16.0.5-16.7.7 (Ausubel, *et al.*, *eds.* John Wiley & Sons (1996)). Briefly, a fusion protein vector is constructed for insertion into *E. coli*. This vector will generally contain a selectable marker gene sequence, a controllable transcriptional promoter (such as *lac*, *trp* or tac), other translational control sequences such as appropriately positioned ribosome-binding site and initiator ATG and one or more polylinker sequences to facilitate insertion of the fusion protein gene in the correct orientation within the vector. This vector will also contain a "carrier sequence" that encodes a carrier protein; this carrier sequence is inserted into the expression vector 5' of the gene to be expressed. The carrier sequence generally encodes a protein that is strongly expressed in *E. coli*. The carrier sequence will provide the necessary signals for proper expression and the expressed protein will contain an N-terminal region encoded by the carrier sequence. The carrier

sequence can encode an entire protein, such as β-glucuronidase and ß-galactosidase.

A fusion protein according to the invention generally comprises an antibody or antibody fragment as described above and an enzyme. The production of antibodies and antibody fragments according to the invention are described above, as are the methods for isolating nucleic acid sequences that encode such antibodies and antibody fragments. The enzymes of the fusion proteins according to the invention include cytoplasmic enzymes, including *E. coli* cytoplasmic enzymes such as *E. coli* β-glucuronidase. The *E.coli* β-glucuronidase gene (uid A) has been cloned, sequenced, and expressed as a fusion with the *E. coli lacZ* promoter and coding region by Jefferson et al. (PNAS vol. 83, pp. 8447 - 8451, 1986). The enzyme β-glucuronidase is capable of cleaving ß-glucuronides to toxic counterparts. When fused to an antigen-binding polypeptide, an enzyme in a fusion protein according to the invention can cleave a non-toxic compound (known also as a prodrug) into its active, and toxic, form.

A recombinant expression vector encoding a fusion protein according to the invention can be expressed in *E. coli* using transformation and culturing techniques that are well known in the art, using techniques such as those described by Ausubel, *supra*, pp. 1.8.1-1.8.8 and 16.0.5-16.4.2. Briefly, calcium chloride can be used to transform *E. coli* with foreign DNA. Those of skill in the art will recognize that the following factors can influence the success of the transformation: harvesting bacterial cells during logarithmic growth phase, keeping cells on ice during transformation and avoiding prolonged exposure of cells to calcium chloride. Electroporation is also well-known in the art as an acceptable transformation method for *E. coli*. Those of skill in the art will recognize the need to vary the electric pulse strength and length to optimize transformation and to add sufficient DNA for transformation.

Thioredoxin reductase-deficient *E.coli*

An *E. coli* strain which is deficient in thioredoxin reductase, for example the strain AD 494, can form disulfide bridges in the cytoplasm and thus enzymes which are naturally secretory, for example alkaline phosphatase, can be expressed intracellularly (Derman et al., Science, vol. 262. 1744 - 1747, 1993). As used in this specification, a thioredoxin reductase-deficient *E. coli* strain is one which (1) has TRR activity that is eliminated or greatly reduced compared to the wild type strain and (2) is capable of producing cytoplasmic proteins with disulfide bonds. *E. coli* strains with (1) mutations in the *trxB* gene (which codes for TRR) and (2) that are capable of producing cytoplasmic proteins with disulfide bonds are included in the present invention.

A TRR-deficient *E.coli* strain can be isolated by selection methods known in the art. For example, it is known that alkaline phosphatase (AP) requires two intrachain disulfide bonds that are required for AP to retain its enzymatic activity. If AP is expressed in *E. coli* without its signal sequence, it remains in the cytoplasm and its disulfide bonds are not formed. Any mutant *E. coli* which produces enzymatically active, cytoplasmic AP is able to create disulfide bonds in the cytoplasm and is hence TRR-deficient. Thus, one assay to select for TRR-deficient strains is as follows. AP can function as a phosphomonoesterase. In *E. coli*, it is known that fructose-1,6-bisphosphatase (*fbp*) is a cytoplasmic phosphomonoesterase that is required for the growth of *E. coli* on gluconeogenic carbon sources such as glycerol. If an AP gene (without its signal sequence) that is expressed in an *fbp*- mutant will cause such a mutant to grow on glycerol, then the AP gene has enzymatic activity and the mutant is capable of creating disulfide bonds. An *E. coli* mutant selected in this way should be TRR-deficient. Jefferson, *supra*, describes the use of an *fbp*- mutant to select for mutant *E. coli* that allow for cytoplasmic disulfide bond formation. These

fbp- mutants would only grow on glycerol if expression of a signal-sequenceless AP gene was induced with IPTG (isopropyl *thio*-ß-D-galactopyranoside).

A second selection step can also be employed. AP can also perform the phosphoserine dephosphorylation function of the enzyme encoded by the *E. coli serB* gene. This is the final step in serine biosynthesis. Thus, if a deletion is introduced into the *serB* gene and induction of the signal-sequenceless AP gene restores the *E. coli* to Ser+, this indicates the presence of active cytoplasmic AP and hence the formation of disulfide bonds in the cytoplasm.

Purification of fusion proteins

The fusion proteins of the invention can be purified by various techniques well-known in the art. Following culturing of the transformed *E. coli* harboring the fusion protein gene, the cells are typically disrupted, suspended and pelletted to remove cell debris. Fusion protein can be purified from the supernatant. Numerous methods for ion-exchange chromatography and purification according to size are well characterized in the prior art and one of skill in the art could readily select appropriate purification techniques based on the properties of the fusion protein produced according to the invention. See Ausubel, *supra*, pp. 16.6.1-16.8.14. Affinity chromatography is also a technique well-known in the art. Anti-idiotype affinity chromatography is useful for purification of fusion proteins with an antibody or antibody fragment component. Briefly, the antiidiotypic antibody is ligated to CnBr-activated Sepharose to create the affinity matrix. The fusion-protein-containing supernatant is exposed to the affinity matrix and the fusion protein is eluted, typically using a pH gradient.

The following examples illustrate but do not limit the invention.

Exampl 1

Construction of a dicistronic expression vector without signal sequences

A) Cloning the *E. coli* β-glucuronidase from *E. coli* RR1:

The DNA sequence encoding *E. coli* β-glucuronidase was amplified by PCR from the *E. coli* strain RR1 using the primers E.c.β Gluc. for (AAG CTT TCA TTG TTT GCC TCC CTG CTG CGG) and E.c.β Gluc. back (TCT AGA CCA TGG TAC GTC CTG TAC AAA CCC CA), and cloned into the vector P bluescript II KS (Stratagene, La Jolla, Calif.) by way of the Xba I and Hind III sites (Fig. 1).

B) Cloning of VH/CH1 and linker Mab BW 431/26 in front of *E. coli* β-glucuronidase:

The antibody variable domain VH and the constant domain CH1 were amplified by PCR from an HC-human-β-glucuronidase cDNA construct, using the primers Fab HC for (GAA TTC CAT GGA ACC AGA ACC AGA ACC GAG CTC AAC TCT) and Fab HC back (TCT AGA TAA CGA GGG CAA AAA ATG GAG GTC CAA CTG CAG), and cloned into vector p bluescript II KS by way of the Xba I and Eco RI sites (Fig. 2). See Bosslet et al., Br. J, Cancer, 65, 234 - 238, 1992 and Güssow & Seemann, *Methods in Enzymology*, 203:99-121 (1991). Bosslet describes the construction of the vector comprising a cDNA sequence encoding humanized VH (derived from the VH gene of Mab BW431) and CH1, fused to the gene for human ß-glucuronidase that was used in this step. Briefly, the fusion gene comprises the human IgG promoter region and signal peptide exon, the humanized version of the VH gene of Mab BW431 (described in Güssow, *supra*), and the CH1 exon of human IgG3.

After the DNA sequence had been verified, a DNA fragment which was isolated by digesting with XbaI and NcoI was ligated into the vector from cloning step A which had been cut with XbaI and NcoI (Fig. 3).

C) Cloning the Fab BW 431/26 - *E. coli* β-glucuronidase into pTrc 99:

An Xba I/Hind III fragment containing the human heavy chain construct fused to *E. coli* β-glucuronidase was ligated to the light chain of Mab BW 431/26, which was present in the expression vector pTrc99. See Amann et al., Gene 69, 301, 1988 (Fig. 4). The resulting construct has the structure: promotor (trc) - Shine Dalgarno sequence (SD) - VK/CK Mab BW 431/26 - SD -VH/CH1 Mab 431/26 - *E. coli*-β-gluc.-transcription termination signal (Fig. 5). This construct was used to transform *E. coli* AD 494 using standard transformation techniques known in the art. See, *e.g.*, Ausubel, *supra* pp. 1.8.1-1.8.8.

Example 2

Expression in AD 494

Overnight cultures of AD 494, harboring pTrc dicistr. Fab-*E. coli*-β-gluc., described in Example 1, were diluted 1:10 and incubated at 25°C until they reached an OD_{600} of 0.7. After inducing with 1 mM IPTG for 19 - 22 hours, the cells were incubated on ice for 1-1.5 hours. After the cells had been pelletted and resuspended in 10 ml of PBS, pH 7.2, per liter of culture volume, they were disrupted in a French press at 1000 - 1500 Psi. The disrupted cell suspension was clarified at 20,000 rpm in an SS-34 rotor and the supernatant was employed for the subsequent investigations.

Example 3

Purification of the fusion molecule by affinity chromatography.

The disrupted cell suspension supernatant, which had been clarified by passing it through a 2 μm filter, was purified by affinity chromatography. The fusion protein was allowed to bind to an anti-idiotypic monoclonal antibody (BW 2064(34) (6 mg of Mab/ml of CnBr-activated Sepharose 4B) as described in Bosslet, *British J. Cancer* 65:234 (1992) and Bosslett, *British J. Cancer* 63:681 (1991). The fusion protein bound to the affinity column was eluted by pH shifting (pH 7.2 - pH 5.0). The peak of antibody was eluted with pH 5.0. (Table 1). The eluate

was then concentrated by means of ultrafiltration (Filtron Macrosep. Omega NMWL:30 KD). The cell disruption suspension supernatant, the void volume, the concentrated eluate and the filtrate from the ultrafiltration were analyzed by SDS-PAGE. The band appearing at about 97 KD corresponds, as regards its molecular weight, to the expected fusion protein comprising the heavy chain moiety of the antibody and the *E. coli* glucuronidase. The band appearing at about 70 KD represents endogenous β-glucuronidase which has been purified concomitantly, during the affinity chromatography, due to the formation of heterotetramers (see below) between expressed heavy chain/β-glucuronidase and endogenous β-glucuronidase.

Example 4

TSK 3000 gel chromatography

The native molecule was examined by TSK 3000 gel chromatography and its molecular weight was found to be 450 KD. Since the glucuronidase forms a tetramer in the native state, the observed molecular weight corresponds to that which is to be expected theoretically. This step is described in detail below.

400 ng of a fusion protein, which had been purified by anti-idiotype affinity, in 25 µl were chromatographed on a TSK gel G 3000 SW XL column (TOSO HAAS, Cat. No. 3.5WxN3211, 7.8 mm x 300 mm) in an appropriate mobile phase (PBS, pH 7.2, 5 g/l maltose, 4.2 g/l arginine) at a flow rate of 0.5 ml/min. The Merck Hitachi HPLC unit (L-400 UV detector, L-6210 intelligent pump, D-2500 chromato-integrator) was operated at about 20 bar, the optical density of the eluate was determined at 280 nm, and 0.5 ml fractions were collected, using an LKB 2111 Multisac fraction collector, and subsequently analyzed in an enzyme activity specificity test (Example 5). The experiment is depicted in Fig. 6. Based on elution pattern of the the molecular weight markers (indicated by arrows), the functionally active Fab-*E. coli* β-

glucuronidase fusion protein was determined to have a molecular weight of about 450 KD.

Exampl 5

Demonstration of the antigen-binding properties and the enzymatic activity

The ability of the Fab-*E. coli* β-glucuronidase fusion protein to bind specifically to the Mab 431/26-defined epitope on CEA (carcino-embryonic antigen) and simultaneously to exert the enzymic activity of the β-glucuronidase was demonstrated in an enzyme activity specificity test. This assay is carried out as described below:

- Polystyrene (96-well) microtiter plates (U shape, Type B, supplied by Nunc, Order No. 4-60445) are incubated with purified CEA (1-5 µg of CEA/ml, 75 µ of this per well) or with GIT mucin (same amount as CEA) at R.T. overnight.
- The non-adsorbed antigen is removed by aspiration and washed 3 x with 0.05 M tris/citrate buffer, pH 7.4.
- The microliter plates are left to stand at R.T. with the opening facing downwards on cellulose overnight.
- The microtiter plates are incubated with 250 µl of 1% strength casein solution in PBS, pH 7.2, per well (blocking solution) at 20°C for 30 minutes.
- During the blocking, the substrate is made up. The amount of substrate depends on the number of supernatants to be assayed. The substrate is made up fresh for each assay.
- Substrate: 4-methylumbelliferyl β-D-glucuronide (Order No.: M-9130 from Sigma), 2.5 mM in 200 mM sodium acetate buffer, pH 5.0, with 0.01% BSA.
- The blocking solution is removed by aspiration, and in each case 50 µl of BHK call supernatant which contains the fusion protein are loaded onto the microtiter plate coated with CEA or GIT mucin (that is to say the sample volume required is at least 120 µl).

- Incubation at R.T. is then carried out for 30 minutes.
- The plates are washed 3 x with ELISA washing buffer (Behring, OSEW 96).
- The substrate is loaded in (50 µl/wall) and incubated at 37°C for 2 hours. The plate is covered because of the possibility of evaporation.
- After 2 hours, 150 µl of stop solution are pipetted into each well (stop solution = 0.2 M glycine + 0.2% SDS, pH 11.7).
- Evaluation can now be carried out under a UV lamp (excitation energy 380 nm) or in a fluorescence measuring instrument (Fluorosean 11, ICN Biomedicals Cat. No.: 78-611-00).

See also EP-A-0 501 215 A2. The test determines the liberation of 4-methylumbelliferone from 4-methylumbelliferyl-β-glucuronide by the β-glucuronidase moiety of the fusion protein after the fusion protein has bound to the antigen by its Fab moiety. The fluorescence values which were determined are given as relative fluorescence units (FU) (Table 1). This assay demonstrates that the fusion protein elicits significant liberation of methylumbelliferone in the CEA-coated plates.

PEM (polymorphic epithelial mucin)-coated plates served as the control. In these plates, the fluorescence signal was always less than 30 FU.

Table 1

	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
FU, Disrupted cell suspension 1:3	8183	8149	7531	6631	4560	2509	1019	421.9
FU, void volume 1:1	6548	5231	3222	1477	525.2	214	86.19	46.29
FU, pH 5.0 eluate 1:3	7782	7571	6360	4239	1983	815.7	302	113.9
FU, pH 5.0 eluate Ultraconcent. 1:10	7904	8106	8036	7153	5802	3618	1651	665.7
FU pH 5.0 eluate Ultrafiltrate 1:1	74.65	172.7	90.23	52.30	38.84	25.79	23.51	19.39